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þÿ B a n i , M a r t i n a

2018-07

þÿ B a n i , M , U r o i , K , L e b o a P a v u n c , A , N o v a k , J , Z o r i , K , D u r g o
þÿ J a m n i k , P , K a z a z i , S , K a z a z i , S , R a d o v i , S , S c a l a b r i n , S , H y
J & Kos , B 2018 , ' Characterization of S-layer proteins of potential probiotic starter culture Lactobacillus brevis SF9B isolated from sauerkraut ' , LWT-Food Science and Technology , vol. 93 , pp. 257-267 . https://doi.org/10.1016/j.lwt.2018.03.054

http://hdl.handle.net/10138/300244
https://doi.org/10.1016/j.lwt.2018.03.054

Accepted Manuscript

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PII: S0023-6438(18)30278-0

DOI: 10.1016/j.lwt.2018.03.054

Reference: YFSTL 6983

To appear in: *LWT - Food Science and Technology*

Received Date: 19 December 2017

Revised Date: 16 February 2018

Accepted Date: 20 March 2018

Please cite this article as: Banić, M., Uroić, K., Leboš Pavunc, A., Novak, J., Zorić, K., Durgo, K., Petković, H., Jamnik, P., Kazazić, Saš., Kazazić, Snjež., Radović, S., Scalabrin, S., Hynönen, U., Šušković, J., Kos, Blaž., Characterization of S-layer proteins of potential probiotic starter culture *Lactobacillus brevis* SF9B isolated from sauerkraut, *LWT - Food Science and Technology* (2018), doi: 10.1016/j.lwt.2018.03.054.

Characterization of S-layer proteins of potential probiotic starter culture *Lactobacillus brevis* SF9B isolated from sauerkraut

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Abstract

S-layers represent the simplest biological membranes developed during the evolution and are one of the most abundant biopolymers on Earth. Current fundamental and applied research aim to reveal the chemical structure, morphogenesis and function of S-layer proteins (Slps). This is the first paper that describes the Slps of certain *Lactobacillus brevis* strain isolated from sauerkraut. The whole genome sequence (WGS) analysis of the *L. brevis* SF9B strain uncovered three genes encoding the putative Slps, but merely one, identified as similar to the SlpB of *L. brevis* ATCC 14869, was expressed. Slp-expressing SF9B cells exhibited increased survival in simulated gastrointestinal

(GI) conditions and during freeze-drying. Their survival in stress conditions was additionally enhanced by microencapsulation, especially when using alginate with gelatine as a matrix. Thus prepared cells were subjected to simulated GI conditions and their mortality was only 0.28 ± 0.45 log CFU/mL. Furthermore, a correlation between the high surface hydrophobicity and the remarkable aggregative capacity of SF9B strain was established. The results indicate a prominent role of Slps in adhesion to mucin, extracellular matrix (ECM) proteins, and particularly to Caco-2 cells, where the removal of Slps utterly abolished the adhesiveness of SF9B cells for 7.78 ± 0.25 log CFU/mL.

Keywords: *Lactobacillus brevis*; probiotics; Slps; adhesion; microencapsulation

1. Introduction

Traditionally produced sauerkraut has already been ascertained as a good source of autochthonous lactic acid bacteria (LAB) which fulfil all technological requirements for application as functional starter cultures. In our previous research, the autochtonous strain *Lactobacillus plantarum* L4, isolated, identified and characterised in our Laboratory, was successfully used in combination with *Leuconostoc mesenteroides* LMG 7954, for the controlled fermentation of cabbage heads, where it allowed lowering of NaCl concentration from 4.0% to 2.5% (w/v), considerably accelerated the fermentation process by 14 days, and improved the product quality (Beganović, et al., 2011a). Autochthonous LAB strains also possess substantial probiotic potential established by their successful survival in simulated GI conditions, adhesion to Caco-2 cells and antibacterial activity against potentially pathogenic microorganisms (Beganović, et al., 2014). The autochthonous strain *Lactobacillus brevis* SF9B was isolated from brine sampled on the 22nd day of the spontaneous fermentation of the high quality Croatian white cabbage *Brassica oleracea* var. *capitata* cultivar Varaždinski, and verified to possess S-layer proteins (Slps), which could ensure advantageous

probiotic potential for the carrying strain. S-layers are paracrystalline bidimensional arrays of protein monomers, fully covering the cell surface of several Gram-positive and Gram-negative bacterial species and archaea during all stages of growth (Gerbino, Carasi, Mobili, Seradell, & Gómez-Zavaglin, 2015). They are metabolic expensive products which may be involved in determining cell shape and cell division, but also may act as protective coats, promoters for cell adhesion, molecular sieves, molecule and ion traps, antifouling coatings, virulence factors in pathogenic organisms (Sleytr, Schuster, Egelseer, & Pum, 2014), and as a scaffold for the external display of other proteins or glycoproteins (Klotz, O'Flaherty, Goh, & Barrangou, 2017). Slps have a significant role as adhesins, which interact with different moieties in the intestinal tissue, and as immunomodulators and protective molecules under environmental stressful conditions (Beganović, et al., 2011b; Uroić, et al., 2016). Moreover, Slps can be used as the carriers of antigens or other important molecules, and therefore are good candidates for health-related applications (Hynönen & Palva, 2013). The aim of this work was to identify the Slps and to evaluate their impact on the probiotic potential of the autochthonous strain *Lactobacillus brevis* SF9B; a possible probiotic starter culture candidate for sauerkraut production.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains employed in this study are listed in **Table 1**. *Lactobacillus* strains and test-microorganisms *E. coli* 3014 and *S. enterica* serovar Typhimurium FP1 were deposited at -80 °C in MRS (de Man Rogosa Sharpe; Difco, Detroit, MI, USA) and BHI (Brain Hearth Infusion; Biolife, Milano, Italy) broth, respectively, supplemented with 15% (v/v) glycerol. The strains were deposited in the Culture collection of the Laboratory for Antibiotic, Enzyme, Probiotic and Starter Cultures Technology, Faculty of Food Technology and Biotechnology, University of Zagreb (CIM-FFTB).

Before every experimental procedure, all the strains were subcultured twice in an appropriate medium under growth conditions listed in **Table 1**.

Since S-layer-deficient lactobacilli mutants are very difficult or impossible to create by recombinant DNA technologies (Hynönen & Palva, 2013), *Lactobacillus plantarum* D13 was used as S-layer-deficient reference strain which naturally coaggregates with *L. brevis*, but does not express Slps (Uroić, et al., 2016). *Lactobacillus helveticus* M92 was used as S-layer-carrying reference strain. Both isolates were used as reference strains in our previous work (Uroić, et al., 2016).

2.2. WGS and identification of genes encoding Slps

The Nextera DNA Library Preparation Kit (Illumina, San Diego, CA, USA) was used for the library preparation. Quantification and quality were tested using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The library was processed with the Illumina cBot and sequenced on the MiSeq2500 (Illumina, San Diego, CA) pair-end with 300 cycles per read, producing 4,980,944 million of reads. The CASAVA 1.8.2 version of the Illumina pipeline was used to process raw data. Raw reads were quality trimmed at both ends with erne-filter v1.4.3 using default parameters and minimum read length of 50 bp, in order to remove low quality bases and preserve only the high quality part of the read (Del Fabbro, Scalabrin, Morgante, & Giorgi, 2013). Adapters ligated to 3' end of each molecule during library preparation were removed with cutadapt (Martin, 2011) using default parameters but -O5 -n2 -m50. Only the relevant part of the read was passed on further analysis. The trimmed reads were *de novo* assembled with CLC Genomics Workbench v7.0. Contigs were classified as belonging to *L. brevis* when receiving the best blastn v2.2.27 hit (Altschul, Gish, Miller, Myers, & Lipman, 1990) with minimum e-value 1e-05 in the NCBI nt database. RAST server, which identifies protein-encoding, rRNA and tRNA genes, assigns functions to the genes, and predicts which

94 subsystems are represented in the genome (Aziz, et al., 2008), was used for the annotation. The
95 assembled contigs were compared with so far identified Slps in the NCBI using the tblastn v2.2.27.

96 **2.3. Treatment with GHCl**

97 The putative Slps were extracted from the surface of *Lactobacillus* cells as described earlier
98 by Uroić, et al., (2016). Following overnight incubation, cells were harvested, washed, resuspended
99 in 5 mol/L guanidine hydrochloride (GHCl), and incubated with shaking at room temperature for 2
100 h. Next, cells were washed to remove residual GHCl. Untreated lactobacilli cells were prepared for
101 the experiments in the same way; only distilled water was used instead of GHCl.

102 **2.4. Detection and identification of Slps**

103 The extraction of Slps from the cell-surface was performed as described in Section 2.3. The
104 extracts were precipitated using the 2D-Clean-up kit (GE Healthcare, Amersham, UK) and subjected
105 to SDS-PAGE and 2D-PAGE, according to Jakava-Viljanen & Palva (2007) and Petelinc, Polak, &
106 Jamnik (2013). Spots from SDS-PAGE and 2D-PAGE gels were cut out and subjected to in-gel
107 digestion by trypsin (Shevchenko, Tomas, Havlis, Olsen, & Mann, 2006). The obtained mixture of
108 tryptic peptides was analysed by the LC-MS analysis (Zhang, Fonslow, Shan, Baek, & Yates, 2013)
109 before the Mascot database search, which uses tandem mass spectrometry data to identify
110 proteins from primary sequence databases (Perkins, Pappin, Creasy, & Cottrell, 1999). The Phyre2
111 automatic fold recognition server (http://www.sbg.bio.ic.ac.uk/phyre2/) was used for predicting
112 the secondary structure of the isolated Slp, whereas the homology modelling of the target Slp
113 protein was performed using the I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-
114 TASSER/) and the data available for *L. brevis* ATCC 14869.

115 **2.5. Survival under stress conditions**

116 For the examination of cell survival during incubation in simulated GI conditions, simulated
117 gastric and small intestinal juices were prepared as described by Kos, Šušković, Goreta, & Matošić,
118 (2000). Overnight cultures of *Lactobacillus* strains were harvested by centrifugation, washed twice,
119 resuspended in simulated gastric juice (pH=2) and incubated for 2 h before cell viability was
120 determined. Immediately after the incubation in simulated gastric juice, cells were harvested by
121 centrifugation, resuspended in simulated small intestinal juice (pH=8) and incubated for 4 h before
122 cell viability was determined.

123 The examination of cell viability during freeze-drying was performed as described by Uroić,
124 et al., (2016). Briefly, bacterial cells grown to late exponential phase were harvested by
125 centrifugation, washed twice and resuspended in phosphate buffered saline (PBS, pH=7) with or
126 without the addition of 10% skim milk (Dukat, Zagreb, Croatia) as lyoprotectant. Suspensions were
127 frozen overnight at -70 °C and freeze-dried in the CHRIST Alpha 1-2 LDplus freeze dryer (Martin
128 Christ, Osterode, Germany) for 30 h. Cell viability in all of the experiments was determined by
129 counting the number of colony forming units (CFU/mL) developing on MRS agar using a standard
130 pour-plate technique.

131 **2.6. Microencapsulation and coating procedures**

132 The microencapsulation of the SF9B cells in sodium alginate was performed according to
133 Gbassi, Vandamme, Ennahar, & Marchioni (2009). Afterwards, microencapsulated cells were
134 immersed in 20 g/L whey protein (Dukat, Zagreb, Croatia) solution and gently shaken for 15
135 minutes. The mixture was filtered to recover the coated microcapsules. Moreover, the solutions of
136 10% (w/v) gelatine (Fisher Scientific, Loughborough, UK) and 2% (w/v) sodium alginate (Fluka,
137 Buchs, Switzerland) were mixed in 2:1 (v/v) ratio (Li, Chen, Cha, Park, & Liu, 2009) and the SF9B cells

were immersed in thus prepared suspension and microencapsulated in 0.1 mol/L $CaCl_2$. Microcapsules were rinsed twice with saline solution and freeze-dried. The entrapped bacteria were released by homogenization in 0.05 mol/L NaH_2PO_4 (pH 8.0, 45 °C) and the number of viable cells was determined by pour-plate method. Subsequently, lyophilised SF9B cells microencapsulated in the gelatine-alginate matrix were subjected to simulated GI conditions as described in Section 2.5.

2.7. Microbial adhesion to solvents (MATS)

Microbial adhesion to solvents (MATS) assay was applied to evaluate the cell-surface characteristics of the probiotic strains through the analysis of microbial cell binding to an apolar solvent, hexane. The assay was performed as initially described by Bellon-Fontaine, Rault, & van Oss (1996) with some modifications introduced by Kos, et al. (2003). Briefly, cells were harvested in the stationary phase by centrifugation, washed twice and resuspended in 0.1 mol/L KNO_3 to approximately 1 x 10^9 CFU/mL. 3 mL of hexane was added to 1 mL of each cell suspension and the mixture was incubated for 10 min at room temperature. Following incubation, a two-phase system was mixed by vortexing and incubated for another 20 min at room temperature to ensure complete separation of the phases. Then, the aqueous phase was removed and its absorbance at 600 nm was measured.

2.8. Autoaggregation and coaggregation assays

The autoaggregation and coaggregation assays were carried out as previously described by Kos, et al. (2003). The overnight grown cultures were harvested by centrifugation, washed twice and resuspended in PBS to obtain viable counts of approximately 1 x 10^9 CFU/mL. For the autoaggregation assay, cell suspensions were incubated at room temperature and the adsorbance at 600 nm was monitored every 1 hour for a period of 5 hours. The coaggregation assay was

performed by mixing 2 mL of each cell suspension of *Lactobacillus* isolates with the equal volume of suspension of *E. coli* 3014, and *S.* Typhimurium FP1, respectively. Control tubes were set up simultaneously, containing 4 mL of each bacterial suspension on its own. The as-prepared mixtures were incubated at room temperature and the absorbance was monitored at different time (0 and 5 h).

2.9. *In vitro* adhesion of *L. brevis* SF9B to mucin, Caco-2 cells and immobilized ECM proteins

In vitro adhesion to mucin (Sigma-Aldrich, St Louis, MO, USA) bound to 96-well polystyrene plates (Maxisorp Nunc, Roskidle, Denmark) was examined according to Vishwanath & Ramphal (1984).

Caco-2 cells were routinely grown in 24-well plates until confluent monolayers were obtained and carefully rinsed three times with PBS (pH 7.4). Two equivalents of 10 mL lactobacilli cultures were routinely cultivated overnight, harvested by centrifugation (4200 g, 10 min) and washed twice in PBS (pH 7.4). One equivalent was treated with 5 mol/L GHCl. Cells were afterwards diluted in medium without antibiotics, supplemented with 10% (v/v) heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS). 1.0 mL aliquot of bacterial suspension (approximately 1 x 10^9 CFU/mL) was added to each well and incubated for 1h at 37 °C in an atmosphere of 5% CO_2. Cells were then washed three times with PBS (pH 7.4) to remove non-adhered bacterial cells. Caco-2 cells were lysed by addition of 0.25% (v/v) Triton X-100 *(*AppliChem*,* Darmstadt*,* Germany*)* solution at 37 °C for 10 min. The adherent bacterial cells were collected and their number was specified by plating on MRS agar plates.

Adherence to individual proteins of the mammalian ECM was tested as described by Antikainen, Anton, Sillanpaa, & Korhonen (2002) with modifications reported in Uroić, et al. (2016).

2.10. Statistical analysis

All the experiments were repeated three times and the results were expressed as means of three independent trials ± standard deviation (SD). Statistical significance was appraised by one-way analysis of variance. Pairwise differences between the means of groups were determined by the Tukey HSD test for post-analysis of variance pairwise comparisons (http://vassarstats.net/test). Statistical differences between groups were considered significant when P values were less than 0.05.

3. Results

3.1. Sequencing of the whole genome and identification of genes encoding Slps

A draft genome sequence of *Lactobacillus brevis* SF9B is available in GenBank under the Accession number NIGJ00000000 (BioProject PRJNA388578, Biosample SAMN07179267). It contains 2,467,947 nucleotides with an overall G+C content of 45.9% in 74 contigs. The information on distribution and categorization of all the annotated genes is shown in **Fig. 1**. Using tblastn v2.2.27 with default parameters, the translated assembly was compared with so far identified Slp amino acid sequences deposited in NCBI. The three regions showed high homology with the following slp sequences of strain *Lactobacillus brevis* ATCC 14869: with SlpB and SlpC in contig 2 (percentage of identities 65% and 89%, respectively) and with SlpD in contig 32 (percentage of identities 99%). The RAST annotation of the assembly corroborated a hypothesis of the presence of genes in these regions. Furthermore, the three predicted SF9B Slp amino acid sequences were multiple aligned with four Slp amino acid sequences of *L. brevis* ATCC 14869 deposited in NCBI (SlpA, SlpB, SlpC and SlpD) using ClustalW (**Supplementary file 1**). The pairwise alignment scores

confirmed similarity of sequences encoded by contig 2_9131-7695 with SlpB (score 64), contig 2_7505-6126 with SlpC (score 89) and contig 32_15226-16467 with SlpD (score 99) (**Fig. 2**).

3.2. Detection and predicted structure of Slps

SDS-PAGE of GHCl-extracted proteins of SF9B strain revealed a 50 kDa protein band indicating the expression of presumed Slp. 2D-PAGE of the same sample revealed a spot with isoelectric point and MW of approximately 10, and 50 kDa, respectively (**Fig. 3**). The spots from both gels were cut out and subjected to LC-MS analysis which, combined with Mascot Database search, identified the protein similar to SlpB of *L. brevis* ATCC 14869 with theoretical MW of 50.9 kDa and pI of 9.54 (**Table 2**). In attempt to predict the secondary structure of isolated Slp, I-TASSER modelling was performed using the data available for *L. brevis* ATCC 14869 (**Supplementary file 2**).

3.3. Protective role of Slps and microencapsulation in stress conditions

Compared to the reference strains, *L. brevis* SF9B demonstrated the highest survival rate in simulated GI conditions. The removal of Slps considerably increased the cell mortality of S-layered strains (**Table 3**). However, the viability of *L. plantarum* D13 cells remained unaltered after the GHCl treatment, with the mortality rates similar to those of S-layer-depleted SF9B and M92 cells. The same protective effect of S-layer was observed through the lyophilisation trial. Although the use of skim milk as a lyoprotectant did not affect the survival of untreated SF9B cells, it significantly ($P < 0.05$) improved the survival of S-layer-depleted cells (**Table 3**).

Additionally, the survival of lyophilised SF9B cells previously microencapsulated in alginate (uncoated or coated in whey proteins) or in the mixture of alginate and gelatine was examined (**Table 4**). The number of viable cells before microencapsulation was 1.15×10^9 CFU/ml. The microencapsulation matrices exhibited different effects on the cell survival during lyophilisation and merely the combination of alginate and gelatine ensured complete protection of the cells, since

significant ($P < 0.05$) difference in the number of viable cells before and after the lyophilisation was not observed in that case. Considering the promising outcome of the alginate/gelatine microencapsulation, thus prepared cells were subjected to simulated GI conditions and the determined cell mortality was only 0.28 ± 0.45 log CFU/mL.

3.4. Cell surface hydrophobicity, aggregation and coaggregation ability of *L. brevis* SF9B

MATS method was performed to assess the role of Slps in the cell surface properties of *L. brevis* SF9B. The comparison of the percentage of affinity towards the hexane, presented in **Fig. 4** revealed the higher affinity of SF9B strain and the reference strain *L. helveticus* M92 for hexane, opposed to the reference strain *L. plantarum* D13. In addition, GHCl treatment considerably ($P < 0.01$) reduced the affinity of S-layered strains from high to low, while it did not influence the moderate adhesion of D13 strain.

According to the results shown in **Fig. 5A**, the examined strain SF9B, demonstrated the highest autoaggregation rate, followed by the reference strain M92. After the GHCl treatment, the autoaggregation ability of SF9B and M92 cells was significantly ($P < 0.01$) lower, unlike that of D13 cells. As seen in **Fig. 5B** & **C**, SF9B strain exhibited the strongest coaggregation with both indicator strains. Moreover, the GHCl treatment negatively affected the coaggregation ability of all strains.

In order to elucidate the potential correlation between the cell-surface hydrophobicity and aggregation of *Lactobacillus* strains, the percentages of autoaggregation and coaggregation were plotted against hydrophobicity values, and the Pearson correlation coefficient was analysed (**Fig 6A**, **B** & **C**). The correlation coefficient was the highest between hydrophobicity and autoaggregation (0.922), although a very strong correlation was observed between hydrophobicity and coaggregation with *S.* Typhimurium FP1 (0.920) and *E. coli* 3014 (0.714), as well. The correlation coefficient between autoaggregation and coaggregation of *Lactobacillus* strains with *E. coli* 3014

250 was 0.764, while exceptionally strong correlation (0.969) was observed between lactobacilli
251 autoaggregation and their coaggregation with *S.* Typhimurium FP1.

252 **3.5. *In vitro* adherence of *L. brevis* SF9B to mucin, epithelial Caco-2 cells and subepithelial ECM**
253 **proteins**

254 The adhesiveness of both S-layer-expressing and S-layer-depleted bacteria to immobilized
255 mucin was also determined (**Fig. 7A**). The untreated SF9B cells demonstrated the strongest binding
256 affinity to mucin, whereas the adhesion of *L. helveticus* M92 and *L. plantarum* D13 reference strains
257 was considerably lower (34.73 ± 7.71% and 54.16 ± 10.33%, respectively). The application of GHCl
258 significantly decreased ($P < 0.01$) the adhesion percentage of both S-layer-expressing strains (80.61
259 ± 5.82% for SF9B and 80.31 ± 7.85% for M92), however not ($P \geq 0.05$) of the S-layer-deficient D13
260 strain (21.10 ± 1.67%).

261 The potential of SF9B strain to adhere to Caco-2 human intestinal cells was also evaluated.
262 As shown in **Fig. 7B**, SF9B demonstrated the strongest *in vitro* adherence. Moreover, GHCl
263 treatment significantly affected the adherence of D13 strain and utterly abolished the adhesion
264 ability of both S-layered strains.

265 In order to examine the role of Slps in the ECM binding, the adhesion of SF9B cells to human
266 fibronectin, laminin and collagen, before and after the GHCl or proteinase K treatment, was studied
267 (**Table 5**.). All the strains successfully adhered to immobilized fibronectin and collagen at different
268 levels, whereas binding to laminin was altogether less effective. In comparison with the S-layered
269 reference strain M92, binding of the untreated cells of both SF9B and the S-layer-deficient D13
270 strain to each ECM protein was less efficient at different extent; 11.42 ± 7.52% and 51.44 ± 7.69%
271 to fibronectin, 23.70 ± 1.16% and 30.47 ± 5.01% to collagen, 65.65 ± 7.99% and 92.45 ± 5.42% to
272 laminin, respectively. Compared with the untreated control, the adhesion of GHCl-treated cells to

fibronectin was significantly ($P < 0.05$) lower, except for the reference strain D13. Moreover, proteinase K treatment nearly completely eradicated the adhesive capacity of all lactobacilli to fibronectin and collagen, whereas it significantly ($P < 0.01$) reduced merely the adherence of the reference strain M92 to laminin.

4. Discussion

Since the health benefits of probiotics are exclusively strain-specific, identification to the strain level is the main prerequisite for screening, selection and identification of novel probiotic strains. In recent years, WGS provided extremely valuable information for bacterial strain typing and allowed the quantification of genome-wide differences between strains through the comparison of nucleotide sequences (Treven, 2015; Tagini & Greub, 2017). The WGS of SF9B strain revealed that it taxonomically belongs to *Lactobacillus brevis* species, usually isolated from a broad spectrum of environments such as fermented foods (Leboš Pavunc, et al., 2012) or the GI tract of humans and animals (Beganović, et al., 2014; Uroić, et al., 2014).

The distribution and categorization of all the annotated genes of SF9B strain revealed that the most abundant groups of genes are included in the carbohydrate and the protein metabolisms; the two most widely represented microorganism functions. Taking into account the abundance of Slps present in the cell wall of SF9B strain, it is not surprising that the third most abundant group of the annotated genes is associated with the cell wall and capsules. The detection of the putative Slp-encoding genes was one of the main goals of WGS of *L. brevis* SF9B. The comparison of the translated assembly with the Slp sequences deposited in NCBI database revealed three positive matches in the two of the annotated contigs of SF9B. The obtained percentages of identities are considered high regarding the low sequence similarity generally observed among *slp* genes (Gerbino, Carasi, Mobili, Seradell, & Gómez-Zavaglin, 2015). The results indicate the presence of

three genes encoding the putative Slps in the analysed genome-homologs of *slpB, slpC* and *slpD* genes of *Lactobacillus brevis* ATCC 14869. The comparison of predicted amino acid sequences of these three putative *L. brevis* Slps with sequences published in NCBI, indicates the subdivision of each sequence into two regions: a conserved N-terminal region and a more variable C-terminal region, which corresponds to so far identified the Slps of *L. brevis* strains (Åvall-Jääskeläinen, et al., 2008).

In an attempt to reveal which *slp* genes are expressed, the Slps of *L. brevis* SF9B were extracted and separated by SDS-PAGE, indicating a 50 kDa protein as a potential Slp. The same sample was then separated with 2D-PAGE. Since the Slps of lactobacilli are highly basic (Åvall-Jääskeläinen, et al., 2008), we cut out a highly expressed spot on 2D gel with the observed isoelectric point of approximately 10 and MW of approximately 50 kDa. Using the LC-MS analysis and the Mascot database search, this spot was identified as homologue of the SlpB (*Lactobacillus brevis* ATCC 14869). Among the three *slp* genes of SF9B strain, only *slpB* was expressed, while *slpC* and *slpD* are considered silent under employed experimental conditions. The obtained results did not provide enough information to make conclusions regarding the secondary and tertiary structure of isolated Slp. However, since that Slp demonstrated similar primary structure to SlpB of *L. brevis* ATCC 14869, presumably their other structures are similar, too. The secondary structure prediction for SlpB of *L. brevis* ATCC 14869, made by Phyre2 server, suggests 6% α-helices, 46% β-strands and 51% of the structure as disordered, which is in correspondence with the literature describing an average of 14% α-helices, 39% extended strands and 47% random coils in these proteins (Hynönen & Palva, 2013; Qamsari, et al., 2017). Additionally, the presence of a conserved N-terminal region with high predicted pI in the *L. brevis* Slps strongly suggests N-terminal cell wall binding domain, while the more variable C-terminal one presumably facilitates the assembly of Slp subunits (Åvall-Jääskeläinen, et al., 2008). As the Slps of *Lactobacillus* strains are very specific

proteins due to their inherent properties, other information on their structure are still rather scarce (Hynönen & Palva, 2013).

Increasing scientific evidences highlight that the enhanced survival in rigorous GI conditions and during the lyophilisation are related to the occurrence of specific Slps present on the surface of some lactobacilli (Beganović, et al., 2011a). Tolerance to low pH and bile salts is a prerequisite for colonisation and metabolic activity of bacteria in the host (Zamfir & Grosu-Tudor, 2014). The important contributors to the viability loss during freeze-drying are osmotic shock and membrane injury resulting from intracellular ice formation and recrystallization occurring under low temperature and low water activity (Li, Chen, Cha, Park, & Liu, 2009). Skim milk is a commonly used lyoprotectant, capable of preventing cellular injury by stabilizing the cell membrane and providing protective coating for the cells (Carvalho, et al., 2004; Zamfir & Grosu-Tudor, 2014). It is assumed that bacterial *slp* genes could be preferentially expressed under unfavourable conditions, forming a mechanical protein barrier which prevents the cells from being directly exposed to their environment (Gerbino, Carasi, Mobili, Seradell, & Gómez-Zavaglin, 2015). The protective role of S-layer, discerned against adverse GI conditions and during lyophilisation in our study, was also observed by other authors (Frece, et al., 2005; Meng, et al., 2014; Uroić, et al., 2016).

Moreover, we analysed the protective role of microencapsulation matrices during lyophilisation, since they have great potential as delivery systems for the preservation of probiotics as biotherapeutics and functional starter cultures. The microencapsulation of SF9B cells in alginate was investigated considering its availability, low cost, simplicity, and suitability for food-related products. Microencapsulated probiotic cells used *per os* must survive the passage through the stomach and intestine to be delivered in the colon in sufficient number to exert their beneficial effects. Therefore, additional protective steps were introduced in the microencapsulation process;

343 e.g. alginate beads were additionally coated in whey proteins known for their good buffering
344 capacity. Although Gbassi, Vandamme, Ennahar, & Marchioni (2009) reported that the whey
345 coating of alginate beads significantly improved the survival of encapsulated bacteria, the same
346 procedure did not significantly ($P < 0.05$) affect the survival of SF9B strain. The use of another
347 protective agent, gelatine, known for its thermally reversible gel-forming ability, membrane-
348 forming ability, biocompatibility and non-toxicity (Li, Chen, Cha, Park, & Liu, 2009), utterly
349 protected SF9B cells during lyophilisation. The protective effect of gelatine was also observed
350 during exposure of lyophilised microencapsulated SF9B cells to simulated GI conditions which is in
351 agreement with Li, Chen, Cha, Park, & Liu (2009) who reported higher stability of microcapsule
352 obtained by gelatine-alginate microencapsulated system compared with plain alginate. It is
353 assumed that gelatine forms a good matrix with anionic polysaccharides such as alginate because of
354 its amphoteric nature.

355 Bacterial cell surface hydrophobicity may influence the growth of bacteria on various
356 substrates, as well as their aggregation, biofilm formation and adherence (van Loosdrecht, Lyklema,
357 Norde, Schraa, & Zehnder, 1987). Therefore, hexane adhesion assay was used to evaluate the cell
358 surface hydrophobicity of SF9B strain before and after the removal of Slps and other proteins non-
359 covalently bound to the cell surface. Since the untreated cells of S-layered strains demonstrated
360 higher affinity towards hexane, their surface is certainly more hydrophobic compared to that of the
361 reference strain D13. The decreased affinity of S-layer-depleted cells towards hexane, supports the
362 assumption that Slps contribute to the cell-surface hydrophobicity, which is in accordance with Van
363 der Mei, van de Belt-Gritter, Pouwels, Martinez, & Busscher (2003) and Rong, et al. (2015). The
364 prevalent hydrophobicity of lactobacilli cell-surface presumably facilitates non-specific adherence.
365 Several studies also reported that the occurrence of proteinaceous material at the cell surface

366 caused higher hydrophobicity, while hydrophilic surfaces were associated with the presence of
367 polysaccharides (Kos, et al., 2003; Firoozmand & Rousseau, 2016).

368 Various studies indicated that LAB can prevent the adhesion of pathogenic bacteria to
369 intestinal mucosa through forming a physical barrier via autoaggregation or by coaggregation with
370 the pathogens (Collado, Meriluoto, & Salminen, 2007; Vlková, Rada, Smehilová, & Killer, 2008).
371 Therefore, *in vitro* evaluation of aggregative abilities was used for the preliminary screening of the
372 adhesion potential of putative probiotic strain SF9B. The S-layered strains demonstrated high
373 autoaggregation ability, since more than 80% of their cells were able to autoaggregate within 5
374 hours of incubation. The removal of Slps progressively decreased ($P < 0.01$) their autoaggregation
375 and coaggregation rates to the level similar to that of GHCl-untreated D13 strain, which
376 demonstrated moderate autoaggregation and low coaggregation ability. Thus, the results strongly
377 imply that Slps are somewhat engaged in the aggregation abilities of probiotic isolates, which is
378 consistent with our previous study (Uroić, et al., 2016). Since the GHCl treatment also reduced the
379 coaggregation ability of the S-layer-deficient strain D13, while it did not affect its autoaggregation
380 rate, plausibly some non-covalently bound coaggregation-associated proteins other than Slps, were
381 degraded by the GHCl as well. Since D13 demonstrated a substantial aggregative capacity,
382 regardless of its S-layer deficiency, we can deduce that Slps are not exclusive bacterial aggregating
383 factors. Other studies also ascertained that even though surface-associated proteins are
384 predominantly involved in aggregation and adhesion, other factors like teichoic or lipoteichoic acids
385 and polysaccharides may interfere (Goh & Klaenhammer, 2010). According to the results,
386 coaggregation capability directly correlates with the autoaggregation phenotype. Coaggregation
387 enables lactobacilli to manipulate microenvironment around the pathogens and to constrain their
388 growth in the gut by releasing antimicrobial substances at their very close vicinity (Reid, et al.,
389 1990). SF9B exhibited a strong coaggregation phenotype which contributes to its potential for

interspecific competition with the pathogens and the colonization of the gut. The assumption that coaggregation is directly associated with adhesion is in accordance with Cesena, et al. (2001), who reported that *Lactobacillus crispatusis* is adhering better to Caco-2 cells than its non-aggregation mutant. Since the strains which exhibited stronger autoaggregation and coaggregation capacity, also demonstrated higher cell surface hydrophobicity, a general correlation among bacterial overall surface features and aggregation capabilities is feasible but ought to be assessed on a case to case basis, which was likewise observed by other authors (Collado, Meriluoto, & Salminen, 2007; Tuo, et al., 2013).

The tissue cells of the GI tract are covered by a layer of mucus that protects the epithelium from physical or chemical injury and pathogen infections, promotes gut motility and provides a habitat and nutrients for the commensal intestinal microflora (Cornick, Tawiah, & Chadee, 2015). Mucus represents the first physical barrier that allochthonous bacteria confront in the gut and therefore, *in vitro* adhesion of SF9B to mucin was examined. The results indicate that adhesion to mucin is mediated by the Slps, since their removal significantly ($P < 0.01$) reduced the adhesiveness of S-layered strains, whereas the effect of the GHCl treatment was hardly discernible in D13 strain which adhered to the bound mucin to a lesser extent, regardless of the GHCl treatment. Lactobacilli adhesion to mucus has also been proposed to be mediated by proteins in other studies (Pretzer, et al., 2005; Lukić, et al., 2012). Although mucin is continuously being renewed and produced by goblet cells, it is also being simultaneously degraded by bacterial and human proteases, trauma, various infections or physical erosion in the gut. Microorganisms may then access the underlying tissue structures and therefore, it was important to investigate if SF9B strain is able to bind to the epithelium or subepithelial structures.

Since Caco-2 cells structurally, morphologically and functionally resemble differentiated enterocytes lining the small intestine, a confluent Caco-2 monolayer was used as an *in vitro* model

414 for predicting the ability of SF9B strain to adhere to the human small intestinal mucosa. The
415 complete loss of adhesive capability, which ensued in S-layered strains immediately after the GHCl
416 treatment, is an indicator that Slps probably mediate adhesion to Caco-2 cells. Intriguingly, the
417 adherence of S-layer-deficient strain D13 was also significantly ($P < 0.01$), however not entirely,
418 inhibited. Thus, it is evident that several other non-covalently-bound cell wall proteins, crucial to
419 the cell-adhesion activity, were simultaneously removed. The successful binding of untreated D13
420 cells to Caco-2 cell line elucidated that surface structures, other than the Slps, e.g. lipoteichoic acid,
421 mediate adhesion in S-layer-non-expressing strains, as stated by Jakava-Viljanen & Palva (2007).

422 Though it seems that the adhesion to ECM proteins is not a primary prerequisite for the
423 colonization and exertion of the beneficial effects of probiotics in the gut, since ECMs are covered
424 by epithelial or endothelial cells and a protective layer of mucus and hence are not directly
425 available for bacterial binding, damaged intestinal surface may expose the ECM and allow
426 undesirable microbial colonization and infection. Since many pathogenic bacteria successfully bind
427 to the ECM components, it was important to assess if SF9B strain exhibits the ECM binding
428 capability, important for preventing pathogenic infections in the damaged gut through the
429 competition with pathogens for the same receptor binding sites (Lorca, Torino, Fontd, & Ljungh,
430 2002; Yadav, et al., 2013). The disparity observed in the adhesion abilities of engaged strains
431 suggests that the ECM binding trait is strain, species and genus specific. The removal of Slps
432 drastically reduced the binding of SF9B and M92 to fibronectin. Thus, the results clearly suggest
433 that the Slps mediate adhesion of S-layer-expressing strains to fibronectin. The *in vitro*
434 adhesiveness of D13 towards any immobilized ECM protein was not significantly disturbed by the
435 GHCl treatment whatsoever. The poor adhesion to the fibronectin and collagen of each S-layered
436 strain pretreated with proteinase K, revealed the proteinaceous nature of adhesion molecules and
437 supports the presupposition that Slps indeed are one of the key adhesins of the bacterial cell.

Moreover, proteinase K treatment radically decreased the adhesion of D13 to fibronectin and collagen, whereas it didn't affect its adhesion to laminin. Therefore, the proteins engaged in the adhesion of S-layer-deficient reference strain D13 to fibronectin and collagen, are presumably covalently-bound to the cell surface. The results of several other studies have also demonstrated that Slps may be responsible for the adhesion capability of *Lactobacillus* strains to ECM (Jakava-Viljanen & Palva, 2007; Uroić, et al., 2016).

In conclusion, the present study indicates that the Slp with the theoretical MW of 50.9 kDa and pI of 9.54 has a role in conveying the *in vitro* survival of *L. brevis* SF9B in stress conditions, aggregation and adhesion to various epithelial and subepithelial structures of the GI tract. Since the examined strain owns desirable technological and probiotic characteristics, it is a suitable contender for further studies to elucidate its full potential and possible application as novel probiotic culture.

Acknowledgements

This work has been supported by the Croatian Science Foundation through the project IP-2014-09-7009. Authors also acknowledge the financial support of the University of Zagreb, Croatia. We also thank to Slovenian Research Agency for partial financing the work through the project no. P4-0116.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*(3), 403-410.

Antikainen, J., Anton, L., Sillanpaa, J., & Korhonen, T. K. (2002). Domains in S-layer protein CbsA of *Lactobacillus crispatus* involved in adherence to collagens, laminin and lipoteichoic acids and self-assembly. *Molecular Microbiology, 46*, 381-394.

Åvall-Jääskeläinen, S., Hynönen, U., Ilk, N., Pum, D., Sleytr, U. B., & Palva, A. (2008). Identification and characterization of domains responsible for self-assembly and cell wall binding of the surface layer protein of *Lactobacillus brevis* ATCC 8287. *BMC Microbiology, 8*(165). doi:10.1186/1471-2180-8-165

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., . . . Zagnitko, O. (2008). The RAST server: rapid annotations using subsystems technology. *BMC Genomics, 9*(75). doi:10.1186/1471-2164-9-75

Beganović, J., Frece, J., Kos, B., Leboš Pavunc, A., Habjanič, K., & Šušković, J. (2011b). Functionality of the S-layer protein from the probiotic strain *Lactobacillus helveticus* M92. *Antonie van Leeuwenhoek, 100*(1), 43-53.

Beganović, J., Kos, B., Leboš Pavunc, A., Uroić, K., Jokić, M., & Šušković , J. (2014). Traditionally produced sauerkraut as source of autochthononus functional starter cultures. *Microbiological Research, 169*(7-8), 623-632.

Beganović, J., Leboš Pavunc, A., Gjuračić, K., Špoljarec, M., Šušković, J., & Kos, B. (2011a). Improved sauerkraut production with probiotic strain *Lactobacillus plantarum* L4 and *Leuconostoc mesenteroides* LMG 7954. *Journal of Food Science, 76*(2), 124-129.

Bellon-Fontaine, M. N., Rault, J., & van Oss, C. J. (1996). Microbial adhesion to solvents: a novel method to determine the electron-donor/electron-acceptor or Lewis acid-base properties of microbial cells. *Colloids and Surfaces B-Biointerfaces, 7*(1-2), 47-53.

Carvalho, A. S., Silva, J., Ho, P., Teixeira, P., Malcata, F. X., & Gibbs, P. (2004). Relevant factors for the preparation of freeze-dried lactic acid bacteria. *International Dairy Journal, 14*(10), 835-847.

Cesena, C., Morelli, L., Alander, M., Siljander, T., Tuomola, E., Salminen, S., . . . Vilpponen-Salmela, T. (2001). *Lactobacillus crispatus* and its non-aggregating mutant in human colonisation trials. *Journal of Dairy Science, 84*(5), 1001-1010.

Collado, M. C., Meriluoto, J., & Salminen, S. (2007). Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. *Letters in Applied Microbiology, 45*(4), 454-460.

Cornick, S., Tawiah, A., & Chadee, K. (2015). Roles and regulation of the mucus barrier in the gut. *Tissue Barriers, 3*(1-2). doi:10.4161/21688370.2014.982426

Del Fabbro, C., Scalabrin, S., Morgante, M., & Giorgi, F. M. (2013). An extensive evaluation of read trimming effects on Illumina NGS data analysis. *PLoS One, 8*(12). doi:10.1371/journal.pone.0085024

Firoozmand, H., & Rousseau, D. (2016). Microbial cells as colloidal particles: pickering oil-in-water emulsions stabilized by bacteria and yeast. *Food Research International, 81*(2016), 66-73.

Frece, J., Kos, B., Svetec, I. K., Zgaga, Z., Mrša, V., & Šušković, J. (2005). Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology, 98*(2), 285-292.

Gbassi, K. G., Vandamme, T., Ennahar, S., & Marchioni, E. (2009). Microencapsulation of *Lactobacillus plantarum* spp in an alginate matrix coated with whey proteins. *International Journal of Food Microbiology, 129*(1), 103-105.

Gerbino, E., Carasi, P., Mobili, P., Seradell, M. A., & Gómez-Zavaglin, A. (2015). Role of S-layer proteins in bacteria. *World Journal of Microbiology and Biotechnology, 31*(12), 1877-1887.

Goh, Y. J., & Klaenhammer, T. R. (2010). Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM. *Applied and Environmental Microbiology, 76*(15), 5005-5012.

Hynönen, U., & Palva, A. (2013). Lactobacillus surface layer proteins: structure, functions and applications. *Applied Microbiology and Biotechnology, 97*(12), 5225-5243.

Jakava-Viljanen, M., & Palva, A. (2007). Isolation of surface (S) layer protein carrying *Lactobacillus* species from porcine intestine and faeces and characterization of their adhesion properties to different host tissues. *Veterinary Microbiology, 124*, 264-273.

Klotz, C., O'Flaherty, S., Goh, Y. J., & Barrangou, R. (2017). Investigating the effect of growth phase on the surface-layer associated proteome of *Lactobacillus acidophilus* using quantitative proteomics. *Frontiers in Microbiology, 8*, 2174. doi:10.3389/fmicb.2017.02174

Kos, B., Šušković, J., Goreta, J., & Matošić, S. (2000). Effect of protectors on the viability of *Lactobacillus acidophilus* M92 in simulated gastrointestinal conditions. *Food Technology and Biotechnology, 38*(2), 121-128.

Kos, B., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., & Matošić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology, 94*(6), 981-987.

Leboš Pavunc, A., Beganović, J., Kos, B., Uroić, K., Blažić, M., & Šušković, J. (2012). Characterization and application of autochthonous starter cultures for fresh cheese production. *Food Technology and Biotechnology, 50*(2), 141-151.

Li, X. Y., Chen, X. G., Cha, D. S., Park, H. J., & Liu, C. S. (2009). Microencapsulation of a probiotic bacteria with alginate-gelatin and its properties. *Journal of Microencapsulation, 26*(4), 315-324.

Lorca, G., Torino, M. I., Fontd, V., & Ljungh, A. A. (2002). Lactobacilli express cell surface proteins which mediate binding of immobilized collagen and fibronectin. *FEMS Microbiology Letters, 206*(1), 31-37.

Lukić, J., Strahinić, I., Jovčić, B., Filipić, B., Topisirović, L., Kojić, M., & Begović, J. (2012). Different roles for lactococcal aggregation factor and mucin binding protein in adhesion to gastrointestinal mucosa. *Applied and Environmental Microbiology, 78*(22), 7993-8000.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet Journal, 17*(1), 10-12.

Meng, J., Zhu, X., Gao, S. M., Zhang, Q. X., Sun, S., & Lu, R. (2014). Characterization of surface layer proteins and its role in probiotic properties of three *Lactobacillus* strains. *International Journal of Biological Macromolecules, 65*, 110-114.

Perkins, D. N., Pappin, D. J., Creasy, D. M., & Cottrell, J. S. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis, 20*(18), 3551-3567.

Petelinc, T., Polak, T., & Jamnik, P. (2013). Insight into the molecular mechanisms of propolis activity using a subcellular proteomic approach. *Journal of Agricultural and Food Chemistry, 61*(47), 11502-11510.

Pretzer, G., Snel, J., Molenaar, D., Wiersma, A., Bron, P. A., Lambert, J., . . . Kleerebezem, M. (2005). Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. *Journal of Bacteriology, 187*(17), 6128-6136.

Qamsari, E. S., Sharifzadeh, Z., Bagheri, S., Riazi-Rad, F., Younesi, V., Abolhassani, M., . . . Yousefi, M. (2017). Isolation and characterization of anti C-Met single chain fragment variable (scFv) antibodies. *Journal of Immunotoxicology, 14*(1), 23-30.

Reid, G., McGroarty, J. A., Domingue, P. A., Chow, A. W., Bruce, A. W., Eisen, A., & Costerton, J. W. (1990). Coaggregation of urogenital bacteria *in vitro* and *in vivo*. *Current Microbiology, 20*(1), 47-52.

Rong, J., Zheng, H., Liu, M., Hu, X., Wang, T., Zhang, X., . . . Wang, L. (2015). Probiotic and anti-inflammatory attributes of an isolate *Lactobacillus helveticus* NS8 from Mongolian fermented koumiss. *BMC Microbiology, 15*(196). doi:10.1186/s12866-015-0525-2

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., & Mann, M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature Protocols, 1*(6), 2856-2860.

Sleytr, U. B., Schuster, B., Egelseer, E. M., & Pum, D. (2014). S-layers: principles and applications. *FEMS Microbiology Reviews, 38*(5), 823-864.

Tagini, F., & Greub, G. (2017). Bacterial genome sequencing in clinical microbiology: a pathogen-oriented review. *European Journal of Clinical Microbiology & Infectious Diseases, 36*(11), 2007-2020.

Treven, P. (2015). Strategies to develop strain-specific PCR based assays for probiotics. *Beneficial Microbes, 6*(6), 887-898.

Tuo, Y., Yu, H., Ai, L., Wu, Z., Guo, B., & Chen, W. (2013). Aggregation and adhesion properties of 22 *Lactobacillus* strains. *Journal of Dairy Science, 96*(7), 4252-4257.

Uroić, K., Nikolić, M., Kos, B., Leboš Pavunc, A., Beganović, J., Lukić, J., . . . Šušković, J. (2014). Probiotic properties of lactic acid bacteria isolated from Croatian fresh sort cheese and Serbian white pickled cheese. *Food Technology and Biotechnology, 52*(2), 232-241.

Uroić, K., Novak, J., Hynönen, U., Pietilä, T. E., Leboš Pavunc, A., Kant, R., . . . Šušković, J. (2016). The role of S-layer in adhesive and immunomodulating properties of probiotic starter culture *Lactobacillus brevis* D6 isolated from artisanal smoked fresh cheese. *LWT-Food Science and Technology, 69*(2016), 625-632.

van der Mei, H. C., van de Belt-Gritter, B., Pouwels, P. H., Martinez, B., & Busscher, H. J. (2003). Cell surface hydrophobicity is conveyed by S-layer proteins-a study in recombinant lactobacilli. *Colloids and Surfaces B-Biointerfaces, 28*(2003), 127-134.

van Loosdrecht, M. C., Lyklema, J., Norde, W., Schraa, G., & Zehnder, A. J. (1987). Electrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion. *Applied and Environmental Microbiology, 53*(8), 1893-1897.

Vishwanath, S., & Ramphal, R. (1984). Adherence of *Pseudomonas aeruginosa* to human tracheobronchial mucin. *Infection and Immunity, 45(1)*, 197-202.

Vlková, E., Rada, V., Smehilová, M., & Killer, J. (2008). Auto-aggregation and co-aggregation ability in *Bifidobacteria* and *Clostridia*. *Folia Microbiologica, 53*(3), 263-269.

Yadav, A. K., Tyagi, A., Kaushik, J. K., Saklani, A. C., Grover, S., & Batish, V. K. (2013). Role of surface layer collagen binding protein from indigenous *Lactobacillus plantarum* 91 in adhesion and its anti-adhesion potential against gut pathogen. *Microbiological Research, 168*(10), 639-645.

Zamfir, M., & Grosu-Tudor, S. S. (2014). Stress response of some lactic acid bacteria isolated from Romanian artisan dairy products. *World Journal of Microbiology and Biotechnology, 30*(2), 375-384.

Zhang, Y., Fonslow, B. R., Shan, B., Baek, M. C., & Yates, J. R. (2013). Protein analysis by shotgun/bottom-up proteomics. *Chemical Reviews, 113*(4), 2343-2394.

Table 1. Bacterial strains used in this study

Bacterial Strain	Cultivation conditions
Lactobacillus brevis SF9B	MRS, 37°C, microaerophilic
Lactobacillus helveticus M92	MRS, 37°C, microaerophilic
Lactobacillus plantarum D13	MRS, 37°C, microaerophilic
Escherichia coli 3014	BHI broth, 37°C, aerobic
Salmonella enterica serovar Typhimurium	BHI broth, 37°C, aerobic

Table 2. Identification of the marked protein on SDS-PAGE and 2D-PAGE gel (Mascot results). Ions score is -10 x log(P), where P is the probability that the observed match is a random event. Individual ions scores > 64 (**A**) and > 60 (**B**) indicate identity or extensive homology (P < 0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

A 1::gi|25989136 **Mass:** 50894 **Score:** 282 **Matches:** 6(3) **Sequences:** 5(3) **emPAI:** 0.21
surface layer protein SlpB [Lactobacillus brevis ATCC 14869 = DSM 20054]

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
213	511.2300	1020.4454	1020.5029	-0.0574	0	25	5.7e+002	8	U	K.QPAWTQYK.I
436	731.8300	1461.6454	1461.7827	-0.1373	1	81	0.0011	1	U	K.LANPGKTEAGLTYK.Q
574	923.8800	1845.7454	1845.8996	-0.1542	1	76	0.0031	1	U	K.TIADTTAYKDATFSVDK.V
607	638.5700	1912.6882	1912.8915	-0.2034	0	39	14	4	U	R.EGDTWVHVVNQNTADTK.A
735	724.2600	2169.7582	2170.0403	-0.2822	1	(58)	0.14	1	U	R.TREGDTWVHVVNQNTADTK.A
737	724.3000	2169.8782	2170.0403	-0.1622	1	64	0.046	1	U	R.TREGDTWVHVVNQNTADTK.A

B 1::gi|25989136 **Mass:** 50894 **Score:** 315 **Matches:** 11(3) **Sequences:** 6(2) **emPAI:** 0.13
surface layer protein SlpB [Lactobacillus brevis ATCC 14869 = DSM 20054]

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
112	441.7300	881.4454	881.4494	-0.0040	0	42	4	2	U	K.TEAGLTYK.Q
119	446.2700	890.5254	890.5073	0.0181	0	48	1.2	1	U	K.VVATTTTAK.N
143	481.7400	961.4654	961.4716	-0.0062	0	43	3.8	5	U	K.EGTLTADQK.S
153	492.2600	982.5054	982.4971	0.0083	0	40	6.1	4	U	K.TIADTTAYK.D
441	731.8900	1461.7654	1461.7827	-0.0173	1	86	0.00013	1	U	K.LANPGKTEAGLTYK.Q
442	488.2700	1461.7882	1461.7827	0.0055	1	(41)	4.5	3	U	K.LANPGKTEAGLTYK.Q
549	923.9200	1845.8254	1845.8996	-0.0742	1	(59)	0.054	1	U	K.TIADTTAYKDATFSVDK.V
550	923.9300	1845.8454	1845.8996	-0.0542	1	61	0.036	1	U	K.TIADTTAYKDATFSVDK.V
551	616.2900	1845.8482	1845.8996	-0.0515	1	(59)	0.058	1	U	K.TIADTTAYKDATFSVDK.V
552	616.2900	1845.8482	1845.8996	-0.0515	1	(36)	11	6	U	K.TIADTTAYKDATFSVDK.V
553	616.2900	1845.8482	1845.8996	-0.0515	1	(35)	13	7	U	K.TIADTTAYKDATFSVDK.V

Table 3. Cell mortality (Δlog CFU/mL) of untreated and GHCl-treated probiotic cultures (*L. helveticus* M92, *L. brevis* SF9B and *L. plantarum* D13) exposed to simulated GI conditions and freeze-drying with (skim milk) or without (PBS) lyoprotectans.

Bacterial strain	Simulated GI conditions*		Freeze-drying in PBS		Freeze-drying in skim milk	
	untreated	GHCl treated	untreated	GHCl treated	untreated	GHCl treated
L. helveticus M92	3.40 ± 0.26^{aw}	7.18 ± 0.22^{ax}	0.66 ± 0.17^{ay}	3.10 ± 0.17^{awz}	0.54 ± 0.21^{ay}	2.72 ± 0.16^{az}
L. brevis SF9B	2.04 ± 0.15^{bw}	8.66 ± 0.28^{bx}	1.03 ± 0.16^{by}	3.03 ± 0.22^{az}	1.03 ± 0.19^{ay}	1.92 ± 0.18^{bw}
L. plantarum D13	7.40 ± 0.48^{cx}	7.38 ± 0.37^{ax}	2.91 ± 0.17^{cy}	3.21 ± 0.13^{ay}	1.92 ± 0.18^{bz}	2.96 ± 0.19^{ay}

*Direct transit from simulated gastric juice (pH=2, t=2 h) to simulated intestinal juice (0.3% bile salts, t = 4 h). Statistical analysis was carried out using ANOVA and the results are reported as mean values of three individual experiments ± standard deviation. ^{abc}Different symbol means statistically significant difference ($P < 0.05$) within the same column. ^{wxyz}Different symbol means statistically significant difference ($P < 0.05$) within the same row between the treatments.

Table 4. Survival (CFU/g) of free *L. brevis* SF9B cells and microencapsulated cells in different shell encapsulation materials, after microencapsulation and freeze-drying.

Treatment	After microencapsulation	After freeze-drying
Free cells*	/	$(3.08 \pm 1.97) \cdot 10^{6a}$
Alginate*	$(3.82 \pm 1.60) \cdot 10^{7abz}$	$(2.79 \pm 0.41) \cdot 10^{6ay}$
Alginate + whey proteins*	$(1.02 \pm 0.42) \cdot 10^{8az}$	$(4.34 \pm 0.93) \cdot 10^{7by}$
Alginate + gelatine*	$(1.54 \pm 0.54) \cdot 10^{7bz}$	$(1.53 \pm 0.35) \cdot 10^{7bz}$

*The number of viable cells before microencapsulation was 1.15×10^9 CFU/mL. ^{ab}Different symbol means statistically significant difference ($P < 0.05$) within the same column. ^{yz}Different symbol means statistically significant difference ($P < 0.05$) within the same row. Statistical analysis was carried out using ANOVA and the results are reported as means ± standard deviation of three independent experiments.

Table 5. Effects of GHCl and proteinase K treatments on the binding of *Lactobacillus* strains (*L. helveticus* M92, *L. brevis* SF9B and *L. plantarum* D13) to immobilized ECM proteins: fibronectin, collagen and laminin.

Strain	fibronectin		collagen		laminin	
	GHCl treated	Proteinase K treated	GHCl treated	Proteinase K treated	GHCl treated	Proteinase K treated
L. helveticus M92	72.64 ± 10.62^{ax}	6.17 ± 4.23^{ay}	44.57 ± 15.02^{az}	17.99 ± 2.98^{ay}	59.20 ± 4.33^{axz}	18.35 ± 6.24^{ay}
L. brevis SF9B	67.33 ± 4.87^{ay}	19.26 ± 4.56^{az}	80.79 ± 15.73^{ay}	19.07 ± 7.57^{az}	72.18 ± 27.54^{ay}	52.71 ± 8.80^{byz}
L. plantarum D13	101.76 ± 2.11^{bx}	9.86 ± 9.72^{ay}	91.88 ± 15.66^{ax}	25.95 ± 7.87^{ayz}	57.59 ± 48.20^{axy}	82.91 ± 10.80^{cxz}

Data are adherence ratio of lactobacilli to ECM proteins = (test/control) x 100 (%). Control: adhesion percentage of corresponding untreated cells. Statistical analysis was carried out using ANOVA and the results are reported as means ± standard deviation of three independent experiments. ^{abc}Different symbol means statistically significant difference ($P < 0.05$) within the same column. ^{xyz}Different symbol means statistically significant difference ($P < 0.05$) within the same row between the treatment.

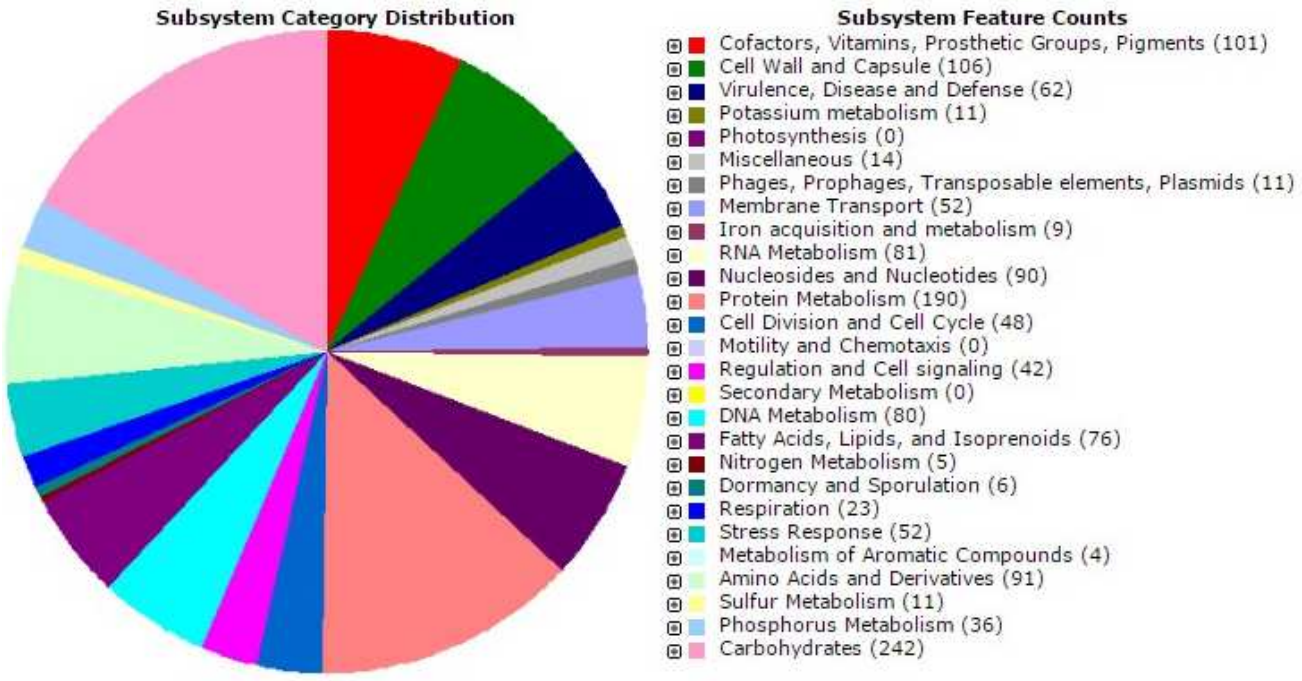

Fig. 1. Information on distribution and categorization of all the annotated genes of *L. brevis* SF9B strain.

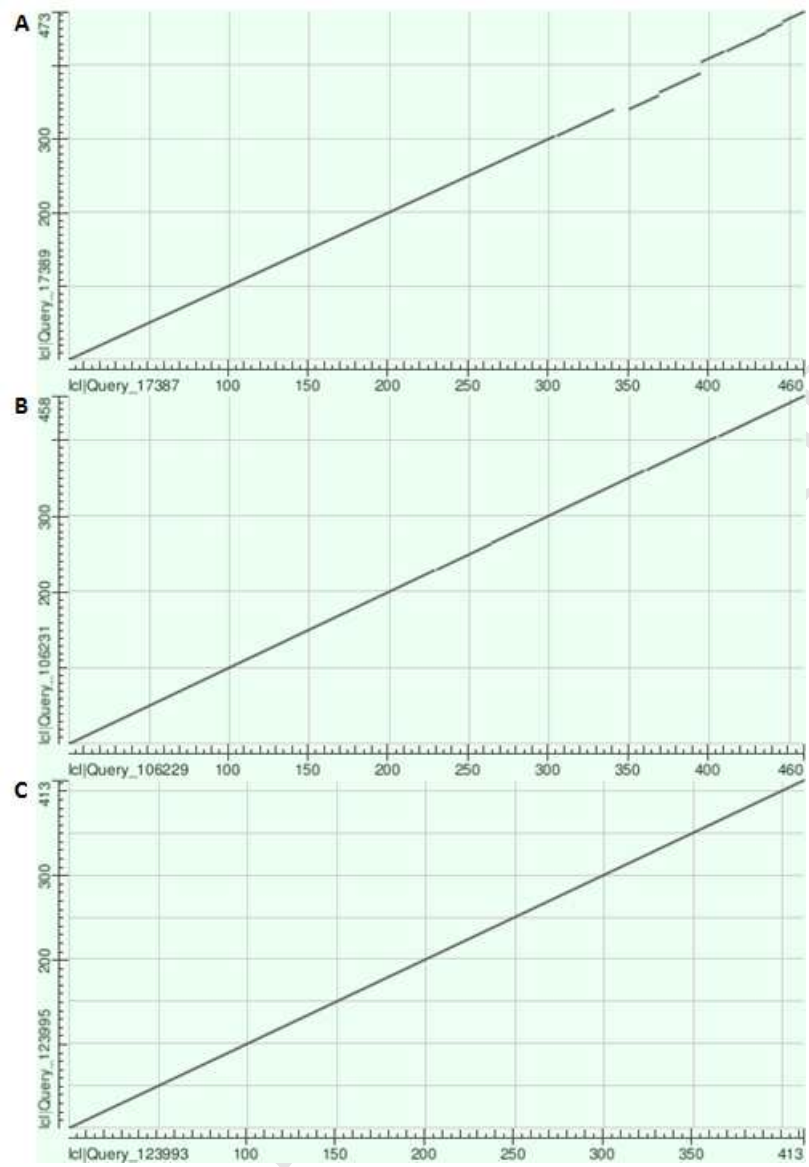

Fig. 2. Visualization of blastP analysis for: **A** slpB vs. contig2 (9131-7695) - Identities: 317/484 (65%), Positives: 359/484 (74%); **B** slpC vs. contig2 (7505-6126) - Identities: 410/461 (89%), Positives: 426/461 (92%); **C** slpD vs. contig32 (15226-16467) - Identities: 411/413 (99%), Positives: 412/413 (99%).

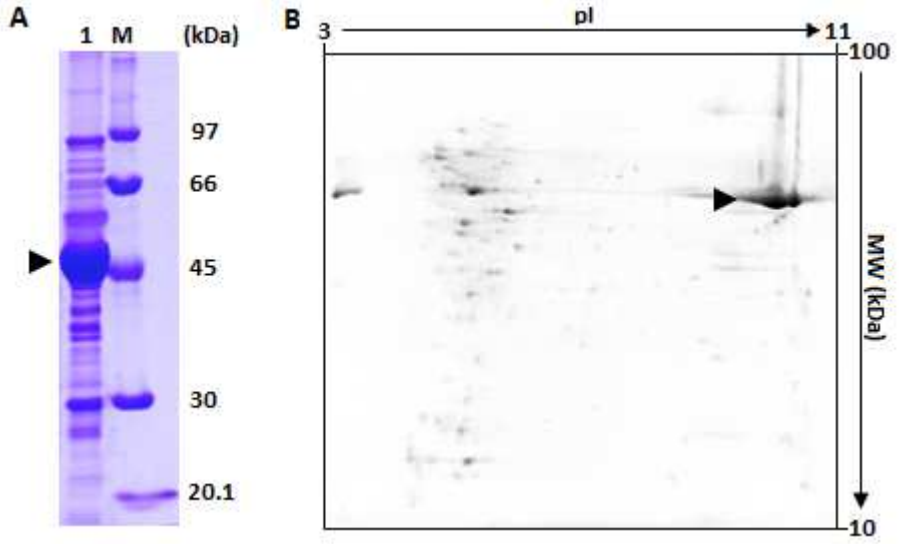

Fig. 3. A SDS-PAGE analysis. Lane 1: surface proteins of *L. brevis* SF9B. Lane M: low molecular weight protein marker standard (GE Healthcare, Amersham, UK). **B** 2D-PAGE analysis of surface proteins of *L. brevis* SF9B. The position of the putative Slp band/spot is marked by an arrow.

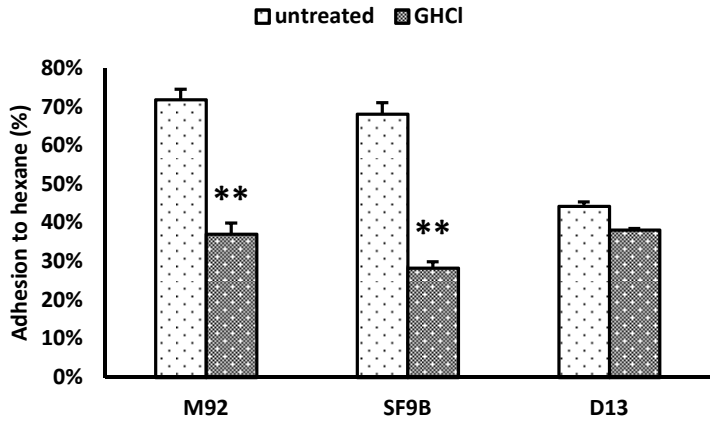

Fig. 4. MATS test: the percentage of adhesion of untreated (▒) and GHCl-treated (▓) probiotic strains (*L. helveticus* M92, *L. brevis* SF9B and *L. plantarum* D13) to hexane. Statistical analysis was carried out using ANOVA. The values are means of three independent experiments and error bars represent standard deviations. **Significantly different ($P < 0.01$) from the untreated control.

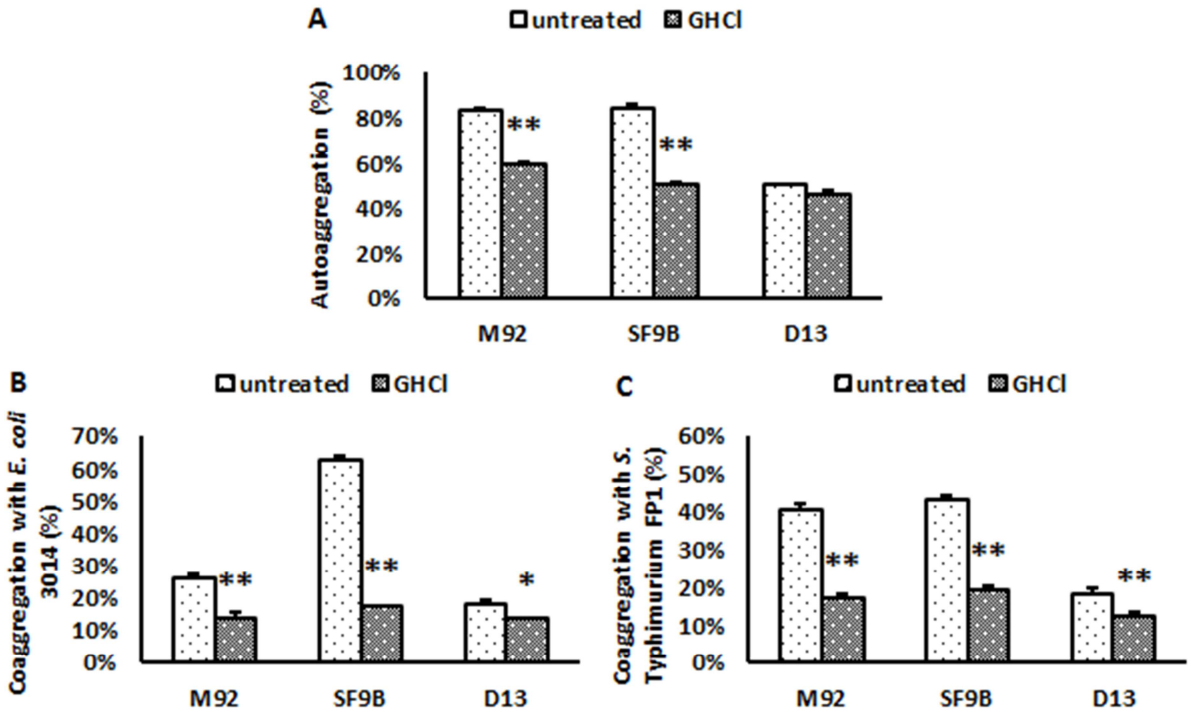

Fig. 5. Comparison of autoaggregation (**A**) and coaggregation percentage of untreated (▒) and GHCl-treated (▓) *Lactobacillus* strains (*L. helveticus* M92, *L. brevis* SF9B and *L. plantarum* D13) with *E. coli* 3014 (**B**) and *S.* Typhimurium (**C**). All the results were achieved by a spectrophotometric assay after 5 h of incubation at room temperature. Statistical analysis was carried out using ANOVA and the results are reported as mean values of three separate experiments ± standard deviation. Asterisks indicate significant differences from the untreated controls at different levels: *P < 0.05, **P < 0.01.

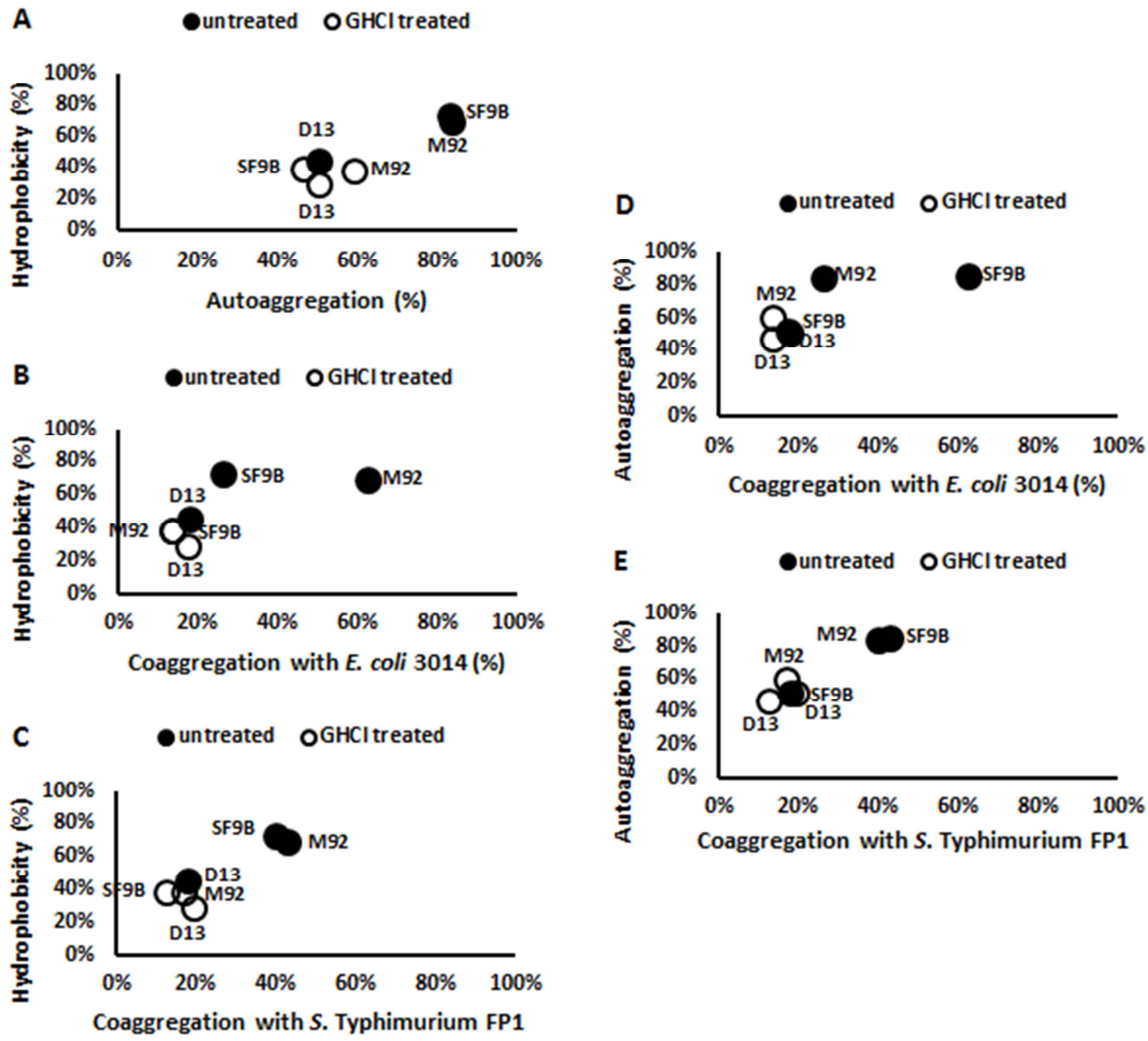

Fig. 6. Relationship between cell-surface hydrophobicity of untreated (●) and GHCl-treated (○) *Lactobacillus* strains (*L. helveticus* M92, *L. brevis* SF9B and *L. plantarum* D13) and: autoaggregation (**A**), coaggregation with *E. coli* 3014 (**B**), coaggregation with *S.* Typhimurium FP1 (**C**). **D** Correlation between autoaggregation and coaggregation with *E. coli* 3014. **E** Correlation between autoaggregation and coaggregation with *S.* Typhimurium FP1. In **A**, **B** and **C**, hydrophobicity is expressed as percentage of bacteria adsorbed by hexane.

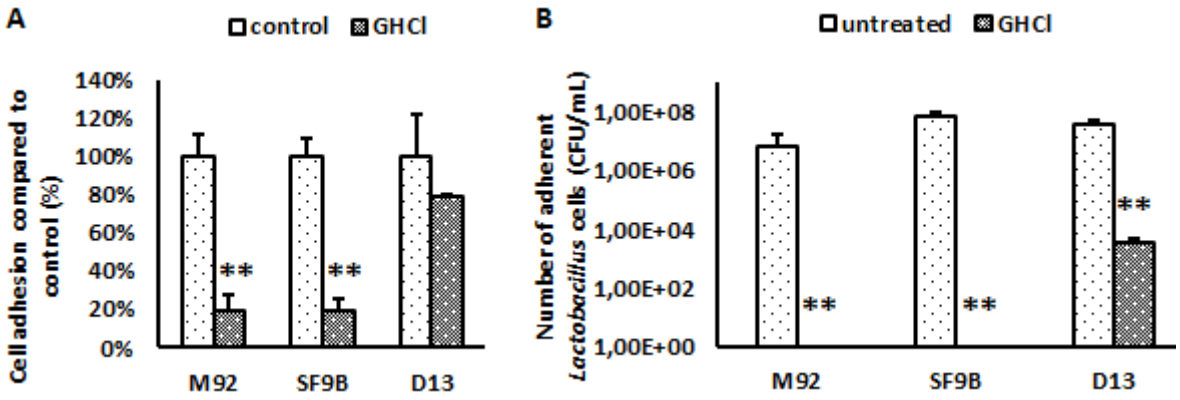

Fig. 7. A Percentage of cell adhesion of GHCl-treated (▓) lactobacilli cultures (*L. helveticus* M92, *L. brevis* SF9B and *L. plantarum* D13) to mucin immobilized to polystyrene plate wells, compared with control cells (set as 100%) which were not treated with GHCl (▒). **B** Adhesion of untreated (▒) and GHCl-treated (▓) probiotic cultures (*L. helveticus* M92, *L. brevis* SF9B and *L. plantarum* D13) to Caco-2 cells. Each adhesion assay was repeated three times. Statistical analysis was carried out using ANOVA and the results are reported as mean values of three individual experiments ± standard deviation. **Significantly different ($P < 0.01$) from the untreated control.

Highlights of the manuscript entitled "Characterization of S-layer proteins of potential probiotic starter culture *Lactobacillus brevis* SF9B isolated from sauerkraut":

- S-layer protein of *L. brevis* SF9B is 65% similar to SlpB of *L. brevis* ATCC 14869
- S-layer protein of *L. brevis* SF9B has theoretical MW of 50.9 kDa and pI of 9.54
- S-layer positively affects probiotic properties of *L. brevis* SF9B
- *L. brevis* SF9B is a potential probiotic starter culture for sauerkraut production